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Identification of Phospholipid as an Essential Part of Bovine Vitamin K-dependent Carboxylase*

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Vitamin K-dependent carboxylase from bovine liver contains phospholipid (primarily phosphatidylcholine), which is essential for its *in vitro* activity. Sepharose-bound carboxylase can be depleted of phospholipids, either by washing the enzyme with detergents or by phospholipase treatment. The enzyme can be reconstituted by adding mixed micelles of phosphatidylcholine and cholate to the Sepharose-bound proteins.

Vitamin K is required for the post-translational carboxylation of a number of glutamic acid-residues in many proteins. Several reviews about this reaction have been published recently (1–3). The carboxylating enzyme system (carboxylase) is obtained from the rough microsomal fraction of hepatic cells (4) and can be extracted therefrom with detergents or bile salts (5). Until now, the purification of carboxylase from this extract has not been accomplished, mainly because the enzyme activity is lost during the purification procedure (5, 6). It has been suggested, therefore, that the enzyme is constituted of at least two components, both of which are required for the carboxylation reaction (7).

We have developed a soluble carboxylating enzyme system from the liver of warfarin-treated cows. This bovine carboxylase was purified more than 100-fold by immunospecific adsorption to antibodies against its endogenous substrate (8). In this paper we present evidence that phospholipids are an essential part of this partially purified carboxylase.

EXPERIMENTAL PROCEDURES

Materials—Buffer A: 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5. Triton X-100, cholate, dithiothreitol, and bovine albumin (essentially fatty acid free), PI,[1] and SPH were obtained from Sigma and vitamin K_1 from Hoffman-La Roche. $NaH^{14}CO_3$ (60 mCi/mmol) and Aquasol 2 were purchased from New England Nuclear and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu from Vega Fox. Phospholipase A_2 (from *Naja naja*), phospholipase C (from *Bacillus cereus*), and phospholipase D (from Savoy cabbage) were prepared as described by Zwaal *et al.* (9). PC was prepared from egg yolk (10) and used for the preparation of phosphatidic acid, PS, PE, and PG (11). Diglycerides were prepared from PC by hydrolysis with phospholipase C and lyso-PC and free fatty acids by hydrolysis with phospholipase A_2. The phospholipids were added to the various phospholipases in the form of mixed micelles, PC and cholate being present in a 1:1 ratio (w/w). The micelles were prepared by evaporating the solvent from the various (phospho)lipids and resuspending the residue in buffer A containing Triton X-100 or cholate as indicated. The suspension was sonicated subsequently until a clear solution was obtained. During the phospholipase treatment the reaction conditions were as described below. The hydrolysis products were separated with the aid of silica thin layer chromatography, using a mobile phase containing chloroform:methanol:NH_4OH:water in a ratio of 90:54:5.5:5.5 (v/v). The lipids were extracted from the silica with a mixture containing equal volumes of chloroform and methanol.

Methods—Carboxylase was prepared and solubilized from the microsomal fraction of the livers of warfarin-treated cows and adsorbed from the solution with antifactor X-Sepharose (8). This solid phase carboxylase (Sp-carboxylase) was used for most of the experiments, and contained 150 μg of protein per ml of Sepharose slurry. The enzyme could be resolubilized from the Sepharose beads by having the carboxylation reaction proceed in buffer A containing 2 mM dithiothreitol, 0.2 mM vitamin KH_2, 4 mM Phe-Leu-Glu-Glu-Leu, 1 mM nonlabeled $NaHCO_3$, and 2 μM factor X. The eluted carboxylase was dialyzed against buffer A before use.

The vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating reaction mixtures (0.25 ml) containing either 0.1 ml of Sp-carboxylase or 10 μg of resolubilized carboxylase and 2 mM dithiothreitol, 0.2 mM vitamin KH_2, and buffer A at 25 °C. When the carboxylation of exogenous substrate was measured, Sp-carboxylase was preincubated in the presence of nonlabeled $NaHCO_3$ (instead of $NaH^{14}CO_3$) for 90 min, washed with buffer A, and then assayed as described above. The reaction was stopped with 2 ml of ice-cold trichloroacetic acid, and the precipitates were washed and counted in Aquasol 2 in a Packard Tri-Carb scintillation counter. The trichloroacetic acid supernatants were degassed at elevated temperatures before counting.

For the treatment of Sp-carboxylase with detergents, 0.5 ml of the Sepharose-bound enzyme was incubated for 30 min at 25 °C in 1.5 ml of buffer A and detergents as indicated. The Sepharose beads were washed subsequently with 20 ml of buffer A containing 2 mM EDTA and 2 mg/ml of bovine albumin and once more with 20 ml of buffer A containing 0.5 mM EDTA. Incubations with phospholipase A_2 and phospholipase C were carried out in buffer A containing 10 mM $CaCl_2$. Incubations with phospholipase D were carried out in 50 mM sodium acetate, 30 mM NaCl, and 50 mM $CaCl_2$, pH 6.3. Sp-carboxylase (0.5 ml) was supplemented with 1.5 ml of buffer A containing 5 IU of phospholipase and incubated for 1 h at 37 °C. After the incubation, the solid phase enzyme was washed in a manner similar to that used after the treatment with detergents.

Phospholipids were extracted from carboxylase according to the method of Bligh and Dyer (12), separated by two-dimensional thin layer chromatography (13), and quantitated by determining the amount of phosphate present in each spot (14). Protein concentrations were determined according to Lowry *et al.* (15). The protein content of Sp-carboxylase was measured after eluting the Sepharose beads with 6 M urea in 2% sodium dodecyl sulfate.

RESULTS

Characteristics of Sp-carboxylase—Sp-carboxylase is prepared by adsorbing solubilized microsomes onto antifactor X-Sepharose. The factor X precursors present in the soluble preparation (8) bind to the antibodies, and because most of the carboxylase is tightly complexed to these factor X precur-

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[1] The abbreviations used are: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SPH, sphingomyelin.

sors (8), the enzyme-substrate complex is bound to the Sepharose beads. The enzyme preparation thus obtained contained 72% protein and 28% phospholipid. With the aid of two-dimensional thin layer chromatography, more than 95% of these phospholipids could be identified as PC.

When reduced vitamin K and $H^{14}CO_3^-$ are added to Sp-carboxylase, the carboxylation of the endogenous substrate starts without delay (Fig. 1*A*). The time course of this carboxylation reaction was not affected by adding 2 mM of the exogenous substrate Phe-Leu-Glu-Glu-Leu (data without peptide not shown), and the reaction rate was constant for about 30 min. The incorporation of $^{14}CO_2$ into the exogenous substrate lagged behind for about 30 min, *i.e.* until about one-half of the endogenous substrate had been carboxylated. After the lag phase, all carboxylase was still firmly bound to the Sepharose beads, but nevertheless, the carboxylation of exogenous substrate started and proceeded with a constant rate for more than 3 h (Fig. 1*A*).

Because we wanted to assay carboxylase in a system that was independent of the variable conditions induced by the preferential carboxylation of the endogenous substrate, we preincubated Sp-carboxylase in the presence of vitamin KH_2 and nonlabeled $NaHCO_3$ and in the absence of the peptide substrate for 90 min at 25 °C. After this period the Sepharose beads were washed and could be used for the carboxylation of the pentapeptide. Now the reaction showed no lag phase and proceeded in a linear way for more than 3 h (Fig. 1*B*). It turned out that during the preincubation no detectable amount of enzyme was lost. The preincubated Sp-carboxylase was used in all experiments mentioned below. We observed three differences between Sp-carboxylase and the vitamin K-dependent carboxylase, present in solubilized microsomes. (*a*) Sp-carboxylase requires reduced vitamin K, whereas carboxylase in solubilized microsomes can also use the nonreduced vitamin (data not shown). Obviously the Sp-carboxylase lacks any K-reductase. (*b*) The K_m of the synthetic pentapeptide is 11 mM in the microsomal extract and 3 mM in Sp-carboxylase. (*c*) Sp-carboxylase is more stable at elevated temperatures than carboxylase in the microsomal extract (Fig. 2). The highest activity was observed at 35 °C, and at this temperature the reaction rate was constant for more than 2 h. When carboxylase was eluted from the Sepharose beads (see under "Experimental Procedures"), the enzyme remained fully active at 35 °C, whereas the nonpurified carboxylase in solubilized microsomes is rapidly destroyed at this temperature.

Effect of Detergents on Sp-carboxylase—The surfactants Triton X-100, cholate, and deoxycholate, which are commonly used for the extraction of carboxylase from the microsomal membranes, strongly inhibited Sp-carboxylase. This inhibition could be prevented by adding a crude extract of microsomal phospholipids to the Sepharose-bound enzyme. Since the bulk of detergent is easily separated from Sp-carboxylase by washing the Sepharose beads, we were able to demonstrate the reversibility of the detergent-induced inactivation (Table I).

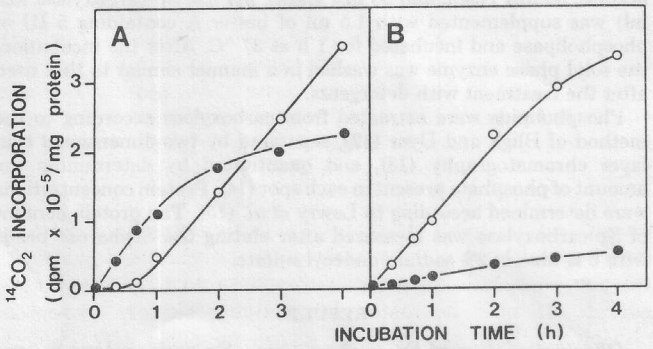

FIG. 1. **The carboxylation of endogenous (●——●) and exogenous (○——○) substrate by Sp-carboxylase.** *A*, Sp-carboxylase was incubated in the presence of vitamin KH_2, $H^{14}CO_3$, and pentapeptide, and the incorporation of $^{14}CO_2$ into the various substrates was measured. *B*, Sp-carboxylase was preincubated at 25 °C in the presence of vitamin KH_2 and nonlabeled HCO_3^- for 90 min, washed with 0.15 M NaCl, and incubated with vitamin KH_2, $H^{14}CO_3$, and peptide substrate as described above.

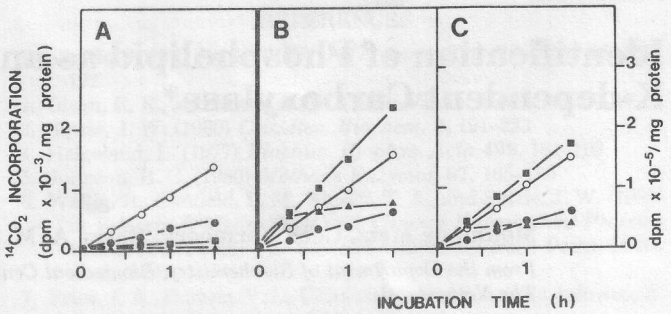

FIG. 2. **Activity of various carboxylase preparations during time course studies at 15 °C (●——●), 25 °C (○——○), 35 °C (■——■), and 45 °C (▲——▲).** *A*, carboxylase in solubilized microsomes; *B*, Sp-carboxylase; *C*, resolubilized carboxylase. The incubation conditions were as described under "Experimental Procedures."

TABLE I

The effect of detergents on the carboxylation rate of Phe-Leu-Glu-Glu-Leu

One ml of Sp-carboxylase was incubated with detergent (as indicated) for 10 min at 30 °C. The Sepharose beads were washed as described under "Experimental Procedures," and the carboxylase activity was measured and expressed as a percentage of the control value (115,000 dpm per mg of protein). The concentration of exogenous phospholipids was 1 mg/ml, and they were added to the enzyme as mixed micelles of phospholipids and cholate. In these micelles the ratio of phospholipid:cholate was 1:1 (w/w). The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Before washing		After washing		
Detergent added	Activity	Phospholipid/protein ratio (w/w)	Carboxylase activity: Minus phospholipids	Carboxylase activity: Plus phospholipids
	% of control		% of control	
None	100	0.40	105	115
Cholate (0.1%)	50	0.38	95	120
Cholate (0.5%)	3	0.06	12	65
Deoxycholate (0.1%)	12	0.25	62	84
Deoxycholate (0.5%)	2	0.04	10	41
Triton X-100 (0.02%)	30	0.30	70	90
Triton X-100 (0.1%)	1	0.02	16	42

At low detergent concentrations, a substantial loss of enzyme activity was observed, but after washing the Sepharose beads with a buffer containing 1 mM EDTA, much of the carboxylating activity was restored, and no significant loss of phospholipids could be measured. At higher detergent concentrations, however, nearly all carboxylase activity was lost. After the washing procedure, about 90% of the phospholipids had been removed, and only 10–15% of the enzyme activity was left. After recombining the washed Sepharose beads with microsomal phospholipids, however, about one-half of the original carboxylase activity could be regained.

The Effect of Phospholipases on Sp-carboxylase—Sp-carboxylase was incubated in the presence of a number of phospholipases, which were subsequently removed by washing the solid phase enzyme with 0.15 M NaCl. In all cases the carboxylating activity was essentially destroyed (Table II), which indicates that either carboxylase requires intact phospholipids

or the degradation products of the endogenous phospholipids (almost exclusively PC) act as inhibitors of carboxylase. The influence of the various split products of PC was, therefore, assessed, and it appeared that the water-soluble choline and cholinephosphate (which are formed by phospholipase D and C, respectively) are readily washed out from the Sepharose beads. Free fatty acids and phosphatidic acid (formed by phospholipase A_2 and D, respectively) are strong inhibitors of carboxylase (Fig. 3), whereas lyso-PC and diglycerides (resulting from the action of phospholipase A_2 and C, respectively) hardly influence the vitamin K-dependent carboxylation. Moreover, when using a washing buffer containing bovine albumin and EDTA, the free fatty acids (as well as lyso-PC) were efficiently removed from the solid phase enzyme. No washing procedure could be developed, however, by which any carboxylase activity was recovered. On the other hand, in all cases the enzyme activity could be partially restored by adding crude microsomal phospholipids. The recovery was about 50% except in the case of phospholipase D, where one of the split products (phosphatidic acid) could not be completely washed out. Since phosphatidic acid is a strong inhibitor of carboxylase (Fig. 3), this low recovery of carboxylase activity is to be expected in this case.

Reconstitution of Phospholipid-depleted Sp-carboxylase with Purified Phospholipids—Sp-carboxylase was depleted from its endogenous phospholipids by incubation with phospholipase C and washed as described above. A number of purified phospholipids was assayed subsequently for their ability to reconstitute the active enzyme (Table III). The neutral phospholipids SPH, PC, PE, and SPH, as well as lyso-PC (the split product that is formed when PC is digested with phospholipase A_2) were able to partially restore the activity of phospholipid-depleted carboxylase. A maximal effect was obtained when the phospholipids were added in a concentration of 0.5–2 mg/ml as mixed micelles with cholate. PC was the phospholipid most effective in restoring the enzyme activity (up to 84%), whereas mixtures of PC and other phospholipids induced a less marked stimulation (data not shown). Negatively charged phospholipids such as PI or PG, as well as phosphatidic acid, did not restore any carboxylating activity. On the other hand, when added to the phospholipid-containing native enzyme, these negatively charged phospholipids strongly inhibited carboxylase. Most probably this inhibition is caused by a rapid exchange between the phospholipids in carboxylase and those in the added micelles. The presence of cholate does not seem to be an absolute requirement for the enzyme, since the removal of cholate by dialysis had no effect on the activity of the reconstituted enzyme preparation.

TABLE II

The effect of phospholipases on the carboxylation rate of Phe-Leu-Glu-Glu-Leu

One ml of Sp-carboxylase was incubated with 1 mg of phospholipase for 1 h at 30 °C. The Sepharose beads were washed as described under "Experimental Procedures," and the carboxylase activity was measured and expressed as a percentage of the control value (115,000 dpm per mg of protein). The concentration of exogenous phospholipids was 1 mg/ml, and they were added to the enzyme as mixed micelles of phospholipids and cholate as described in the legend to Table I. The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Before washing		After washing		
			Carboxylase activity	
Phospholipase used	Carboxylase activity	Phospholipid/protein ratio (w/w)	Minus phospholipids	Plus phospholipids
	% of control		*% of control*	
Phospholipase A_2	0	0.01	4	40
Phospholipase C	1	0.06	6	66
Phospholipase D	0	0.29	1	22

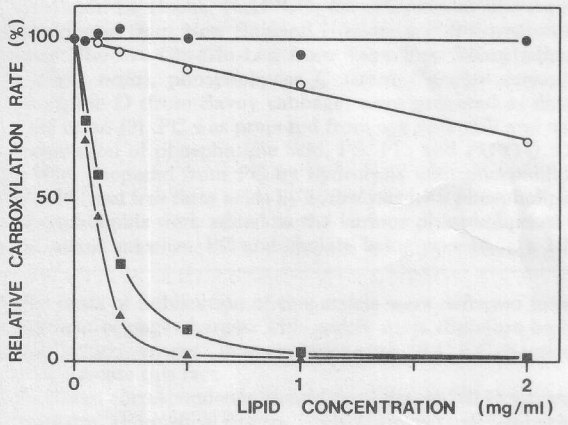

FIG. 3. **Inhibition of Sp-carboxylase by phospholipid degradation products.** Sp-carboxylase was supplemented with mixed micelles of cholate and either lyso-PC (●——●), diglycerides (○——○), phosphatidic acid (■——■), or free fatty acids (▲——▲) in a 1:1 ratio (w/w). Subsequently, the carboxylase activity was measured in the presence of 1 mM EDTA. The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

TABLE III

The dependence of Sp-carboxylase on purified phospholipids

Phospholipid-depleted Sp-carboxylase was prepared with the aid of phospholipase C as described in the legend to Table II. The various phospholipids were added to the enzyme as mixed micelles with cholate in a 1:1 ratio (w/w). The final phospholipid concentration was 1 mg/ml. The results are expressed as a percentage of the control value (115,000 dpm per mg of protein). The incorporation of $^{14}CO_2$ was assessed after 1 h at 25 °C.

Phospholipid added	Carboxylase activity in	
	Sp-carboxylase	Phospholipid-depleted Sp-carboxylase
	% of control	
None	100	5
Crude microsomal extract	104	70
Phosphatidylcholine	110	84
Lyso-PC	98	77
Sphingomyelin	90	50
Phosphatidylethanolamine	50	19
Phosphatidylinositol	3	0
Phosphatidylserine	2	0
Phosphatidylglycerol	2	0
Phosphatidic acid	4	0

DISCUSSION

Sp-carboxylase, which is more than 100-fold purified as compared to the solubilized microsomal extract (8), differed from the latter in that it did not contain vitamin K reductase and in that it was stable at 35 °C. Moreover, it was inactivated by low concentrations of nonionic detergents. Analysis of the Sepharose-bound enzyme showed that it contains about 30% (w/w) phospholipids (amost exclusively PC). When we removed the phospholipid moiety, either by washing carboxylase with detergents or by destroying the phospholipids with various phospholipases, the carboxylase activity had disappeared. It could be restored by adding certain exogenous phospholipids in the form of mixed micelles, with cholate present in a 1:1 ratio (v/v). Vesicles of phospholipids alone were not effective in this respect, probably because in the absence of detergent there is no transfer of phospholipids from the vesicles to the Sepharose-bound proteins. PC turned out to be the most effective in restoring carboxylase activity. This is in agreement with our observation that the natural phospholipid present in Sp-carboxylase is PC. When 1 mM

EDTA was present in the buffers during the separation of the enzyme proteins and phospholipids and during their reconstitution, the recovery of active carboxylase could be greatly improved. As it is well known (16) that integral membrane proteins may aggregate upon removal of their phospholipids, the effect of EDTA might be a prevention of irreversible aggregation or denaturation of carboxylase when the latter is depleted of phospholipids. The fact that phospholipids are an integral part of carboxylase may have implications for the purification of the enzyme. Up to now, several investigators (5, 6) have tried to purify carboxylase, but after fractionating the enzyme in a number of ways they observed that it had lost its biological activity. One of the possible explanations for this phenomenon might be that during these purification procedures the proteins of the enzyme complex are separated from the phospholipid moiety. In order to exclude this pitfall, it seems advisable to add mixed micelles of PC and cholate after each purification step to the fractions when they are assayed for carboxylase activity. When analyzed by sodium dodecyl sulfate gel electrophoresis. Sp-carboxylase contained at least four bands (8), and it is not known whether all these proteins are required for carboxylase activity. The proteins present at this stage of purification are tightly complexed, however, and we have not succeeded in isolating the various components without a complete loss of activity. Whether all these proteins require phospholipid for their proper function cannot be concluded, therefore.

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